EFFECTS OF SUBSTRATE CONCENTRATION AND AGITATION RATE ON BUTANOL PRODUCTION FROM PALM OIL MILL EFFLUENT USING *CLOSTRIDIUM ACETOBUTYLICUM*

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A thesis submitted in fulfillment of the
requirements for the award of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical Engineering and Natural Resources
Universiti Malaysia Pahang

DECEMBER 2010

ABSTRACT

This study is mainly focusing on butanol production from palm oil mill effluent (POME) by anaerobic fermentation using *Clostridium acetobutylicum.* Despite that untreated POME could bring severe effects to environment, POME also can be used as the main substrate due to abundant supply and it potentiality to be utilised by saccharolytic clostridia in butanol fermentation. Reinforced Clostridia Medium (RCM) was functioned as control medium. This study was also to investigate the growth profile rate and the consumption of glucose by *C. acetobutylicum* during fermentation for 72 hour at 37°C. Fermentation was carried out in 250 mL Schott bottle at a working volume of 150 mL. Other parameters were kept constant at pH 5.8 for POME, pH 6.8 for RCM and 10% inoculum. The effects of substrate concentration and agitation rate in producing butanol were studied. Substrate concentrations used were 70%, 80% and 90% while for agitation rates were 0 rpm, 100 rpm, 175 rpm and 250 rpm. Butanol produced from the fermentation was analyzed using gas chromatography equipped with flame ionization detector (GC-FID). Growth profile rate of *C. acetobutylicum* in POME and RCM were measured using UV-Vis spectrophotometer. Glucose concentration was measured from the calculation of the amount of glucose consumed by dinitrosalicylic acid (DNS) method which monitored using UV-Vis spectrophotometer. This experiment were started by clostridia cultivation and then followed by fermentation medium preparation, inoculum preparation, fermentation process for 72 hours and fermentation product analysis. The highest butanol yield in POME was 0.3485 g/L at 70% concentration and 175 rpm while maximum butanol production was produced in 90% RCM at 175 rpm which was 0.5034 g/L. In conclusion, lots of hard work and precaution steps need to be taken in order to make sure higher butanol can be produced at theoretically substrate concentration which is 90% and agitation rate at 200 rpm.

ABSTRAK

Kajian ini memfokuskan kepada penghasilan butanol daripada bahan buangan kilang minyak sawit (POME) oleh *C. acetobutylicum* melalui fermentasi anaerobik. Selain faktor POME yang tidak dirawat akan mendatangkan kesan buruk terhadap alam sekitar, POME boleh digunakan sebagai substrat utama dalam fermentasi kerana ianya dihasilkan dalam jumlah yang banyak dan mempunyai potensi untuk digunakan dalam fermentasi butanol. 'Reinforced Clostridia Medium' (RCM) bertindak sebagai media kawalan. Kajian ini juga dilakukan untuk mengkaji kadar profil pertumbuhan dan pengambilan glukosa oleh *C. acetobutylicum* semasa proses fermentasi selama 72 jam pada suhu 37ºC. Proses ini dijalankan di dalam botol Schott dengan isipadu sebanyak 150 mL. Parameter lain seperti pH 5.8 untuk POME, pH 6.8 untuk RCM dan kepekatan 'inoculum' sebanyak 10% dikekalkan pada keadaan yang optimum. Kesan yang diperolehi terhadap penghasilan butanol daripada kepekatan substrat dan kadar kocakan dikaji. Kepekatan substrat yang digunakan adalah 70%, 80% dan 90% manakala kelajuan yang digunakan adalah 0 rpm, 100 rpm, 175 rpm dan 250 rpm. Butanol yang terhasil daripada fermentasi dianalisis menggunakan GC-FID. Kadar profil pertumbuhan *C. acetobutylicum* dalam POME dan RCM diukur dengan menggunakan spektrofotometer ultra lembayung-nampak. Kepekatan glukosa ditentukan dengan mengira kuantiti glukosa yang digunakan melalui kaedah asid 'dinitrosalicyclic' (DNS) dan diukur menggunakan spektrofotometer ultra lembayung-nampak. Kadar penghasilan butanol tertinggi dihasilkan di POME adalah 0.3485 g/L pada kepekatan substrat 70% dan kelajuan 175 rpm manakala kadar penghasilan butanol yang maksimum terhasil di RCM adalah pada kepekatan substrat 90% dan kelajuan 175 rpm iaitu 0.5034 g/L. Secara kesimpulannya, pelbagai usaha dan langkah-langkah pencegahan perlu diambil untuk memastikan butanol dapat dihasilkan dengan kuantiti yang lebih tinggi pada kepekatan substrat dan kadar kocakan yang sesuai seperti yang dinyatakan dalam teori iaitu pada kepekatan substrat 90% dan kelajuan 200 rpm.

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ABE	-	Acetone-Butanol-Ethanol
BOD	-	Biological Oxygen Demand
C. acetobutylicum	-	*Clostridium acetobutylicum*
DNS reagent	-	Dinitrosalicylic Colorimetric Method
E. coli	-	*Escherichia coli*
GC-FID	-	Gas Chromatography equipped with Flame Ionization Detector
g	-	Gram
L	-	Liter
mL	-	Milliliter
µm	-	Micrometer
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
OD	-	Optical density
POME	-	Palm oil mill effluent
RCM	-	Reinforced Clostridia Medium
S. cerevisiae	-	*Saccharomyces cerevisiae*
UV-Vis	-	Ultra violet vision

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Biofuel derived from biomass has been renewed interest after the so-called oil crisis and tremendous demand in worldwide due to increasing in population. Against a backdrop of rising crude oil prices, depletion resources, political instability in producing countries and environmental challenges, only biomass has the potential to replace the supply of an energy hungry civilisation by producing biofuel.

Butanol fermentation (or also called as acetone, butanol and ethanol fermentation or solvent fermentation), a historical process because it is one of the oldest known industrial fermentations and ranked second to ethanol. Butanol can be produced from a variety of renewable biomass resources as glucose. The most commonly used microorganism which converts these sugars into butanol is *Clostridium acetobutylicum.* Butanol fermentation also is a potential path to upgrade biomass into valuable liquid fuels.

Butanol can be produced from biomass and mineral fuel. Butanol from biomass is denoted as biobutanol to make it differ from butanol produced from petroleum. Primarily used as an industrial solvent, it is now known as other alternative for fuel. Butanol can be used instead of gasoline even in higher degree than ethanol due to its physical properties, economy, safety and because it can be applied without the needs to modify the engine of vehicles. Biobutanol is

environmental friendly as it does not produce sulphur and its by-product is carbon dioxide (CO_2) which is complete combustion.

The key problems associated with the production of biobutanol are the cost of substrate and butanol toxicity or inhibition of the fermenting microorganisms, resulting in a low butanol filter in the fermentation broth. However, recent interest in the production of biobutanol from biomass such as POME has led to the re-examination of acetone-butanol-ethanol (ABE) fermentation. This situation initiated and has sustained interest in identifying and channelling renewable (biomass) raw materials into the manufacture of liquid fuel alternatives because development of such biomass-based power would ensure that new technologies are available to keep pace with society's need for new renewable power alternatives for the future.

Palm oil production is one of the major industries in Malaysia and this country ranks as one of the largest productions in the world. In Malaysia, the total production of crude palm oil in 2008 is 17,734,331 tonnes. However, the production of this amount of crude palm oil results in even larger amounts of palm oil mill effluent (POME). In the year 2008 alone, at least 44 million tonnes of POME was generated in Malaysia. POME consists of water soluble components of palm fruits as well as suspended materials like palm fibre and oil. POME is selected as a substrate in this study because of its abundant supply and low-cost. The availability of supply and cost in previous research by Lee *et al.* (1995) is identified as the main factor in acetone-butanol-ethanol (ABE) fermentation. Compared to other substrates, it has no limitation resources as it produced in huge quantities which make it cheap. Thus, POME is sustainable resources. However, untreated POME have a significant impact on the environment if they are not dealt properly because despite its biodegradability, POME cannot be discharged without first being treated because POME is acidic and has a very high biochemical oxygen demand (BOD) and chemical oxygen demand (COD).

Anaerobic bacteria such as the solventogenic clostridia are capable of converting a wide range of carbon sources (e.g. glucose, galactose, cellobiose, mannose, xylose and arabinose) to fuels and chemicals such as butanol, acetone and

ethanol. Hence, it permits direct fermentation of POME to ABE. This fermentation process is facilitated by *Clostridium acetobutylicum.*

1.2 Problem Statement

This study needs to be conducted due to the undeniable evidence that world is running out of fossil fuel which is non-renewable sources in the next few years. For more than two centuries, the world's energy supply has relied on non-renewable crude oil-derived (fossil) liquid fuels. In addition, worldwide energy demand is bound to increase. Although biofuel as renewable source has been recognised to overcome this problem, many of these alternatives are made from food crops. Several experts have voiced similar concerns that creating biofuel i.e. bioethanol from food crops, such as corn, grains and whey, is considered as stealing food right out of people mouths. Food crisis or shortage also could become crucial due to this.

A number of studies reported that the cost of substrate was identified as the main factor that influences economic viable. Some of fermentation media showed that it is compatible to produce butanol in a huge amount but they are expensive and limited source. Thus, POME is selected as fermentation medium which could help to cut the cost as it is from waste and unlimited source in this country. Hence, producing butanol from abundant waste i.e. POME could replace bioethanol and help to obtain sustainable, inexpensive and suitable substrate.

Untreated POME discharged to water could bring environmental problem due to its high biochemical oxygen demand (BOD), chemical oxygen demand (COD), oil and grease, total solids and suspended solids. Other than that, emissions from the combustion of fossil fuel such as carbon dioxide (CO_2), carbon monoxide (CO), nitrogen oxide (NO_x) and sulphur-containing residues are the principal causes of global warming and its incomplete combustion are harmful to human health.

Butanol is chosen from ABE fermentation because ethanol is regarded to be less superior to butanol as a renewable source of fuel. Ethanol is known for its high volatility because of high vapour pressure and engine modification is needed if want to consume it.

1.3 Objective of Study

This study is proposed with the aim to study the effects of substrate concentration and agitation rate on butanol production from POME in anaerobic condition using *C. acetobutylicum.*

1.4 Scope of Study

The main scopes of study are:

(i) To study the growth profile of *Clostridium acetobutylicum* in POME and Reinforced Clostridia Medium (RCM).

(ii) To study the effects of substrate concentration of 70, 80 and 90% to the butanol production.

(iii) To study the effects of agitation rate at 0, 100, 175 and 250 rpm in producing butanol.

(iv) To study the glucose consumption in fermentation medium.

1.5 Rationale of Study

The main rationale and significance of this study includes:

(i) This study applies concept of ‘waste to wealth’ due to abundant supply of POME as a substrate to yield butanol in huge amount.

(ii) Biobutanol is environmental friendly due to its complete combustion. It is known to contain “green” carbon.

(iii) Another alternative to overcome depleting source petroleum and fossil fuels.

(iv) Butanol produced from POME could help to reduce the production of biofuel from food crops.

(v) Enhance the usage of POME as fermentation media to produce butanol.

CHAPTER 2

LITERATURE REVIEW

2.1 Palm Oil Mill Effluent (POME)

The Malaysian palm oil industry is growing rapidly and quickly becoming a significant agriculture-based industry in this country. Table 2.1 shows that the total productions of crude-palm oil in 2008 and 2009 are 17,734,441 and 16,044,874 tonnes, respectively (MPOB, 2008a, 2009). The palm oil industry provides a source of livelihood to rural families in government land schemes and private small holders, as well as employment opportunities to agricultural workers in estates (Wu *et al.,* 2010). In Malaysia, palm oil is even utilized in the production of biodiesel (palm oil methylester or palm oil diesel) for buses and cars (Wu *et al.,* 2010).

The number of palm oil mills in Malaysia has increased tremendously, i.e. from about 10 mills in 1960 (Ma *et al.,* 1993) to 410 operated mills in 2008 (MPOB, 2008b), in order to meet the crude palm oil demands both logically and internationally. However, the production of such large amounts of crude palm oil results in even larger amounts of palm oil mill effluent (POME) in which cases in the year 2008 alone, at least 44 million tonnes of POME was generated in Malaysia and the figures are expected to rise every year. This alarming figure caused the palm oil industry in Malaysia to be identified as the one generating the largest pollution load in rivers throughout the country (Wu *et al.,* 2010).

Table 2.1 Malaysian production of crude palm oil in 2008 and 2009 (MPOB, 2008a, 2009; Wu *et al.*, 2010)

Month	2008 (tonnes)	2009 (tonnes)
January	1,424,244	1330,195
February	1,227,969	1187,381
March	1,294,710	1275,822
April	1,327,591	1281,852
May	1,457,878	1395,275
June	1,468,921	1447,926
July	1,560,215	1492,958
August	1,600,214	1496,073
September	1,579,442	1557,764
October	1,652,071	1984,036
November	1,658,417	1595,592
December	1,482,769	Data not available
Total	17,734,441	16,044,874

From environmental perspective, fresh POME is a hot and acidic brownish colloidal suspension, characterized by high amounts of total solids (40,500 mg/l), oil and grease (4000 mg/l), COD (50,000 mg/l) and BOD (25,000 mg/l). POME has been identified as one of the major sources of aquatic pollution in Malaysia. The characteristic of a typical POME is shown in Table 2.2. Despite its biodegradability, POME cannot be discharged without first being treated because POME is acidic and has a very high biochemical oxygen demand (BOD). Raw POME is high in BOD and acidic with pH of around 4.0. After treatment, the pH is raised to around 8 and BOD is lowered. In terms of nutrient value, anaerobic sludge of treated POME contains high plant nutrients (Lorestani, 2006).

Table 2.2 Typical characteristics of POME (Ma, 2000; Lorestani, 2006)

Parameter	***Average**	**Metal**	***Average**
pH	4.7	Phosphorus	180
Oil and Grease	4000	Potasium	2270
Biochemical Oxygen Demand (BOD)	25000	Magnesium	615
Chemical Oxygen Demand (COD)	50000	Calcium	439
Total Solids	40500	Boron	7.6
Suspended Solids	18000	Iron	46.5
Total Volatile Solids	34000	Manganese	2.0
Ammonical Nitrogen	35	Copper	0.89
Total Nitrogen	750	Zinc	2.3

*****All in mg/l except pH

It is generally accepted that surplus starchy grains and effluents generated from agro-industrial processes are cheap substrate that could serve as potential fermentation feedstock (Hipolito *et al.,* 2008). In any fermentation process, the cost of the substrate (fermentation medium) will be about 60% of the overall cost (Ross, 1961; Kalil *et al.,* 2003). The availability of an abundant supply of a low-cost, lignocellulosic, agricultural waste substrate is essential if acetone-butanol-ethanol (ABE) fermentation is to become economically viable (Lee *et al.,* 1995). This is due to the cost of the substrate was identified as the main factor that influences the economic viability of ABE fermentation (Lee *et al.,* 1995; Durre, 1998; Ezeji *et al.,* 2004). Furthermore, POME was produced in vast amounts throughout the year could be a kind of sustainable resources (Kalil *et al.,* 2003; Ngan *et al.,* 2004; Wu *et al.,* 2009; 2010).

POME is a thick, brownish liquid with a discharged temperature in the range of 80 to 90^{O}C. In palm oil mills, POME is generated from three major sources, sterilizer condensate, separator sludge and hydrocyclone operation where the broken shells are separated from the kernels (Vijayaraghavan *et al.,* 2005; Takriff *et al.,* 2009). POME consists of various suspended components including cell walls,

organelles and short fibers, a spectrum of carbohydrates ranging from hemicellulose to simple sugar, a range of nitrogenous compound from proteins to amino-acids and free organic acids (Ugoji, 1997; Takriff *et al.,* 2009) and an assembly of minor organic and mineral constituents (Ugoji, 1997; Lorestani, 2006). This entire feature has made POME a potential substrate for ABE fermentation (Somrutai *et al.,* 1996; Kalil *et al.,* 2003, Takriff et *al.,* 2009) and can be utilized by saccharolytic clostridia in ABE fermentation (Kwon *et al.,* 1989; Lee *et al.,* 1995). Such utilization would further increase profitability of palm oil industry besides solving an environmental problem (Kalil *et al.,* 2003).

2.2 Butanol-producing Clostridia

Clostridia have a long history of being employed in several biotechnological processes, for instance, *C. acetobutylicum* play role in the conversion of renewable biomass for butanol production; *C. perfringens* are significant for production of potent toxins such as enterotoxin; *C. botulinum* and *C. tetani* are used for neurotoxins; *C. histolyticum* and *C. oncolyticumto* produce agents for cancer therapy (Gheshlagi *et al.,* 2009) and *C. saccharoperbutylacetonicum* are proven to produce more butanol than *C. acetobutylicum* (Soni *et al.,* 1982). Solvent-producing clostridia were extensively used from the beginning of the 20th century for the industrial production of acetone and butanol and have remained a focus of research because of their potential applications in biotechnology (Keis *et al.,* 2001). Solvent-producing clostridia could produce acetone, butanol and ethanol from several biomass types such as palm oil waste (Lee *et al.,* 1995), domestic waste (Gheshlagi *et al.,* 2009), and abundant agricultural crops (Madihah *et al.,* 2001; Qureshi *et al.,* 2001; Shinto *et al.,* 2008). A number of studies has reported that the production of organic acids, alcohols, and other neutral solvents by the degradation of a wide range of polysaccharides by many species of clostridia (Gheshlagi *et al.* 2009). Saccharolytic mesophilic species that are able to form butyrate, however, are the only species that are capable of producing butanol along with different amounts of acetone, isopropanol, and ethanol (Jones and Woods, 1989; Gheshlagi *et al.* 2009).

Microbial butanol production was first reported by Louis Pasteur in 1861 and developed to an industrial production level by Chaim Weizmann using *Clostridium acetobutylicum* in the early 20th century (Gheshlagi *et al.* 2009). Hence, strains classified as *Clostridium acetobutylicum* were the first industrial cultures to be successfully isolated, patented and used for the large-scale production of solvents from starched-based substrates (Keis *et al.,* 2001). Followed by the switch (in the mid-1930s) to molasses as the preferred fermentation substrate, numerous new solvent-producing clostridial strains were isolated and patented, and each was given a novel species name (Jones and Keis, 1995; Keis *et al.*, 2001). However, none of these saccharolytic industrial strains were recognized as legitimate species, and when the acetone-butanol fermentation process went into decline these names fell into disuse. Subsequently, the majority of these solvent-producing clostridial strains were designated as *C. acetobutylicum* or *Clostridium beijerinckii* (Keis *et al.,* 2001).

C. acetobutylicum is an anaerobic and spore-forming bacterium (Gheshlagi *et al.*, 2009). It also is able to use polymeric substrates such as starch and xylan, but not cellulose, for growth (Durre, 1998). Anaerobic bacteria such as the solventogenic clostridia are capable of converting a wide range of carbon sources (e.g. glucose, galactose, cellobiose, mannose, xylose and arabinose) to fuels and chemicals such as butanol, acetone, and ethanol (Ezeji *et al.,* 2007; Masngut *et al.,* 2007). Hence, *C. acetobutylicum* is a known alcohol-producing microorganism (Alshiyab *et al.*, 2008).

Furthermore, this strain attracts a lot of attention when Finch *et al.,* (2011) reported that by consolidating the functions of waste management, renewable power generation, and solvent production, *C. acetobutylicum* fuel cells have the potential to reduce organic wastes and increases opportunities to convert those wastes to usable energy.

Butanol-producing clostridia such as *C. acetobutylicum, C. beijerinckii and C. pasteurianum* exhibit very similar metabolic pathways. During fermentation, C. acetobutylicum produces three major classes of products: (i) solvents (acetone, ethanol and butanol); (ii) organic acids (acetic acid, lactic acid and butyric acid); (iii)

gases (carbon dioxide, and hydrogen) (Zheng *et al.*, 2009). *C. acetobutylicum* exhibits a biphasic fermentation in which acetate and butyrate are produced initially, known as acidogenesis, followed by a switch to production of the solvents, acetone and butanol (Prescott and Dunn, 1959; Green *et al.*, 1994).

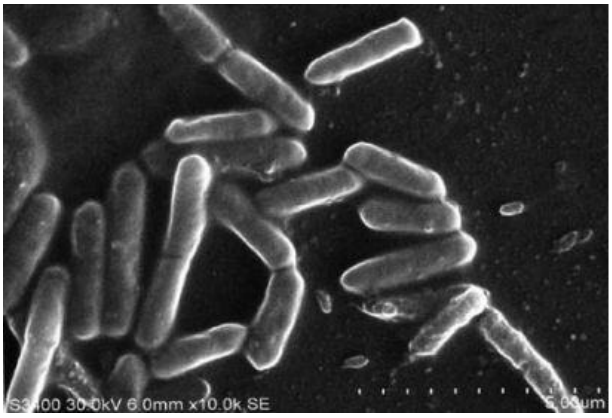

Figure 2.1 Scanning electron microscope (SEM) image of *C. acetobutylicum* cells for ABE production (Ni and Sun, 2009)

2.3 Fermentation

2.3.1 Anaerobic Fermentation

Anaerobic fermentation is the process of fermentation without using any oxygen. Durre (1998) mentioned that the first account of biological butanol synthesis stems from Louis Pasteur. In 1861 he isolated a butyric-acid-forming bacterium and named it *Vibrion butyrique.* This organism was unable to grow in the presence of air; later it became evident that oxygen was the proper toxic compound. This led to the term "anaerobic" to describe this type of metabolism. One of advantages of the anaerobic process is the recovery of the useful matters such as solvents (Hwang et.al. 2004). Anaerobic fermentation is a promising method of sustainable butanol production since organic matter, including waste products, can be used as a feedstock for the process (Alalayah *et al.*, 2009).

2.3.2 Acetone-butanol-ethanol (ABE) Fermentation

A great number of studies were performed in order to improve the process and fermentative process became competitive with chemical synthesis by the middle of the 20th century. Its application, however, declined during the 1950s and was overtaken by cheaper petrochemical-based processes by 1960 (Tashiro *et al.,* 2004; Kobayashi *et al.,* 2005; Gheshlagi *et al.* 2009). Furthermore, end-product inhibition, low product concentration and large volumes of fermentation broth, the requirements for large bioreactors, in addition to the high cost involved in generating the steam required to distil fermentation products from the broth largely contributed to the decline in fermentative acetone-butanol-ethanol (ABE) production (Ishizaki *et al.,* 1999). In the 1980s the reduced supply and escalating price of petroleum rekindled interest in fuel production by anaerobic bacteria including ABE fermentation by various clostridial species (Gheshlagi *et al.,* 2009). This is also due to a worldwide desire to identify and improve alternative but renewable sources of fuels as a safeguard against depleting reserves of fossil fuels have rekindled research into finding ways that would enhance solvent production by the ABE fermentation (Ishizaki *et al.,* 1999).

At present, considerable research has been conducted on the type of ABE fermentation system (Tashiro *et al.,* 2004), including batch culture (Qureshi and Blaschek, 1999; Ishizaki *et al.,* 1999; Tashiro *et al.,* 2004) or fed-batch culture (Ezeji *et al.,* 2004; Tashiro *et al.,* 2004) integrated with a butanol removal process, and continuous culture with concentrated cell mass or immobilized cell mass (Tashiro *et al.,* 2004). In previous studies, the yields of butanol to glucose were under 30%, and the residual glucose concentrations in broth were very high. To date, a highly efficient butanol production system has not yet been established (Tashiro *et al.,* 2004). New possibilities for more sustainable solvent production via ABE fermentation with less expensive substrates have been proposed. For instance, lignocellulosic materials such as domestic organic waste (Gheshlaghi *et al.,* 2009) or fibrous corn wastes (Qureshi *et al.,* 2006; Gheshlaghi *et al.,* 2009) can be used for ABE fermentation.

The metabolic pathways of solvent-producing clostridia consist of two distinct characteristic phases, namely, acidogenesis and solventogenesis (Green *et al.*, 1994; Badr *el al.*, 2001; Kalil *et al.* 2003; Tashiro *et al.*, 2004; Shinto *et al.*, 2008). Typically, during acidogenesis, cell growth is exponential and products are acetic acid and butyric acid with ATP formation. Accumulation of these organic acids results in a decrease in the pH of the broth. During solventogenesis, cell growth enters the stationary phase and the above organic acids are reutilized and acetone, butanol and ethanol are produced. This reutilization of organic acids results in a pH increase of the broth. It is reported that organic acid production is enhanced at higher pH, while solvents are mainly produced at lower pH (15 – 18). On the other hand, since the addition of organic acids to the growth medium has been shown to stimulate solvent production and protect against the degeneration of ABE-producing clostridia, it is suggested that organic acids in broth trigger a metabolic shift from acidogenesis to solventogenesis although the exact mechanism is still unknown. Thus, we noted that butanol could be produced effectively at lower pH by feeding organic acids such as acetic acid or butyric acid. Presently, there is no report on this feeding method in ABE fermentation (Tashiro *et al.*, 2004).

The production of ABE by solvent-producing strains of *Clostridium* was one of the first large-scale industrial fermentation process developed (Kalil *et al.*, 2003). Shinto *et al.* (2008) then mentioned that results of the simulation suggested that *C. saccharoperbutylacetonicum* N1-4 has a robust metabolic network in acid- and solvent-producing pathways (Shinto *et al.*, 2008).

2.4 Butanol

Butanol is a higher alcohol with a four carbon atom structure and a general formula of $C_4H_{10}O$. Butanol can be produced from biomass and from mineral fuel. The butanol from biomass is conventionally denoted as biobutanol despite the fact that it has the same characteristics as the butanol from petroleum (Shapovalov and Ashkinazi, 2008).